

MINIREVIEWS

Oxidation of Reduced Inorganic Sulfur Compounds by Bacteria: Emergence of a Common Mechanism?

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THE SULFUR-OXIDIZING PROKARYOTES

Biological oxidation of hydrogen sulfide to sulfate is one of the major reactions of the global sulfur cycle. Reduced inorganic sulfur compounds (referred to below as sulfur) are exclusively oxidized by prokaryotes, and sulfate is the major oxidation product. Sulfur oxidation in members of the *Eukarya* is mediated by lithoautotrophic bacterial endosymbionts (44).

The sulfur-oxidizing prokaryotes are phylogenetically diverse (Fig. 1). In the domain *Archaea* aerobic sulfur oxidation is restricted to members of the order *Sulfolobales* (21, 58), and in the domain *Bacteria* sulfur is oxidized by aerobic lithotrophs or by anaerobic phototrophs. The nonphototrophic obligate anaerobe *Wolinella succinogenes* oxidizes hydrogen sulfide to polysulfide during fumarate respiration (41). The ecology, physiology, and biochemistry of sulfur-oxidizing bacteria have been reviewed previously. The neutrophilic chemolithotrophic bacteria have been reviewed by Kelly et al. (31, 32, 35) and Takakuwa (63), the acidophilic sulfur-oxidizing bacteria have been reviewed by Harrison (23) and Pronk et al. (46), and the molecular genetics of *Acidithiobacillus ferrooxidans* has been reviewed by Rawlings and Kusano (49). The sulfur metabolism of phototrophic bacteria has been reviewed by Brune (9, 10) and Trüper and Fischer (65). The physiology and genetics of both phototrophic and lithotrophic sulfur-oxidizing prokaryotes have been discussed recently (18).

Prokaryotes oxidize hydrogen sulfide, sulfur, sulfite, thiosulfate, and various polythionates under alkaline (57), neutral, or acidic conditions (23). Aerobic sulfur-oxidizing prokaryotes belong to genera like *Acidianus* (18), *Acidithiobacillus* (36), *Aquaspirillum* (19), *Aquifex* (25), *Bacillus* (2), *Beggiatoa* (60), *Methylobacterium* (15, 34), *Paracoccus*, *Pseudomonas* (19), *Starkeya* (33), *Sulfolobus* (58), *Thermithiobacillus* (36), *Thiobacillus*, and *Xanthobacter* (19) and are mainly mesophilic. Phototrophic anaerobic sulfur-oxidizing bacteria are mainly neutrophilic and mesophilic (10, 65) and belong to genera like *Allochrochromatium* (formerly *Chromatium* [26]), *Chlorobium*, *Rhodobacter*, *Rhodospseudomonas*, *Rhodovulum*, and *Thiocapsa* (9). Lithoautotrophic growth in the dark has been described for *Thiocapsa roseopersicina*, *Allochrochromatium vinosum*, and

other purple sulfur bacteria, as well as for purple nonsulfur bacteria like *Rhodovulum sulfidophilum* (formerly *Rhodobacter sulfidophilus*) (24), *Rhodocyclus genatinosus*, and *Rhodospseudomonas acidophila* (39, 56). This capacity may be based on related biochemical mechanisms of sulfur oxidation in lithotrophic and phototrophic bacteria.

Autotrophic bacteria fix carbon dioxide either via the reductive pentose phosphate cycle or via the reductive tricarboxylic acid cycle. Methylotrophic bacteria fix formaldehyde either via the ribulose monophosphate route or via the serine pathway (16, 61). Reductant released from sulfur oxidation is used in lithotrophic bacteria for aerobic respiration and carbon dioxide reduction, while in anaerobic phototrophic bacteria reductant is used mainly for carbon dioxide fixation. Sulfur oxidation by methylotrophic bacteria has been observed upon growth with methylated sulfur compounds, such as dimethyl sulfide (34). Few reports have focused on the sulfur-oxidizing potential of these organisms (15, 16, 61, 62) due to the major interest in their methylotrophic characteristics.

Two groups of sulfur-oxidizing lithotrophic bacteria have been distinguished previously; members of one group are able to utilize polythionates, and members of the other group are not able to do this (18, 35). On the basis of physiological and biochemical data, at least two major pathways have been proposed for different sulfur-oxidizing bacteria: (i) the sulfur oxidation pathway and (ii) the S_4 intermediate pathway involving polythionates (35). Here we discuss the *sox* gene cluster of *Paracoccus pantotrophus* and the biochemistry and functions of the encoded proteins. On the basis of our analysis, together with available genomic data, we concluded that different metabolic reactions merge into a common mechanism in lithotrophic and phototrophic sulfur-oxidizing bacteria.

THE SULFUR-OXIDIZING SYSTEM OF *P. PANTOTROPHUS*

***sox* gene cluster.** *P. pantotrophus* (48) is a gram-negative, neutrophilic, facultatively lithoautotrophic bacterium that is able to grow with thiosulfate or with molecular hydrogen as an energy source and with a large variety of carbon sources. The gene cluster of *P. pantotrophus* coding for sulfur-oxidizing ability (*Sox*) comprises at least two transcriptional units with 15 genes. Seven genes, *soxXYZABCD*, code for proteins essential for sulfur oxidation in vitro. These genes and *soxFGH* are induced by thiosulfate (11, 20, 42, 51a, 69, 70). Four open

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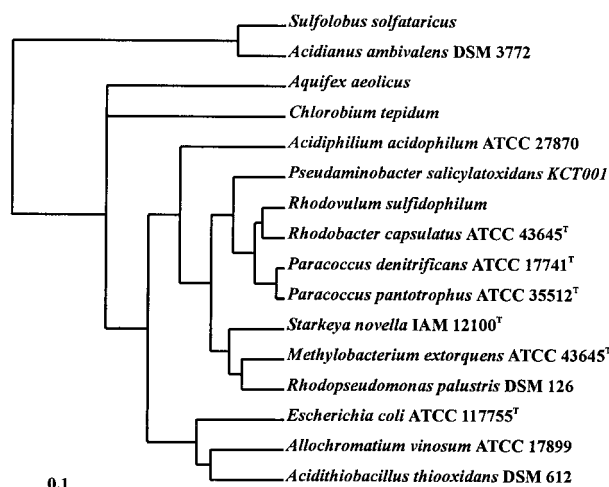


FIG. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis of *P. pantotrophus* and different sulfur-oxidizing bacteria. Bar = 1 inferred nucleotide change per 100 nucleotides.

reading frames (ORFs), ORF1, ORF2, and *shxVW*, are located upstream of *soxX* (Fig. 2), and the *shxVW* ORFs are cotranscribed (F. Bardischewsky and C. G. Friedrich, submitted for publication).

The Sox proteins of *P. pantotrophus* are located in the periplasm, as shown by selective extraction of periplasmic proteins (20), and signal peptides have been predicted for all of the proteins except SoxZ (Table 1). The Sox proteins are transported to the periplasm either via the Sec system or via the Tat system. Twin arginine motifs in the signal peptides are diagnostic for protein transport via the Tat system (6, 8, 68) and are predicted for SoxY, SoxB, SoxC, SoxF, SoxG, and possibly SoxH (Table 1). The SoxY and SoxZ proteins do not contain a prosthetic group, redox center, or metal (20), and SoxZ is likely to be cotransported through the cytoplasmic membrane by SoxY in hitchhiker fashion, as previously shown for the heterodimeric hydrogenases of *Escherichia coli* (51) and *Ralstonia eutropha* (7).

ORF1 predicts a transcriptional regulator of the ArsR family, and ORF2 predicts a periplasmic thioredoxin. Both of these ORFs are oriented divergent to the *sox* gene cluster (Fig. 2). *shxV* predicts a protein with six transmembrane channel-forming helices and a conserved cysteine at helices 1 and 4. ShxV is structurally related to CcdA of *P. pantotrophus*, which is involved in cytochrome *c* biogenesis. CcdA mediates transport of reductant from the cytoplasm to the periplasm for rereduction of the periplasmic apoprotein prior to heme addition. Disruption of *ccdA* results in a complete deficiency of *c*-type cytochromes, which in turn affects metabolic reactions involving *c*-type cytochromes, including sulfur oxidation (4). Disruption of *shxV* by the Ω -kanamycin interposon disables mutant GBQV so that it cannot grow lithotrophically with thiosulfate and with molecular hydrogen. Cytochrome *c* formation is not affected in GBQV, demonstrating that ShxV has a function distinct from that of CcdA. Addition of cysteine to the medium restores growth of strain GBQV with hydrogen but not growth of this strain with thiosulfate. However, the thiosulfate-oxidizing activity of mixotrophically grown GBQV

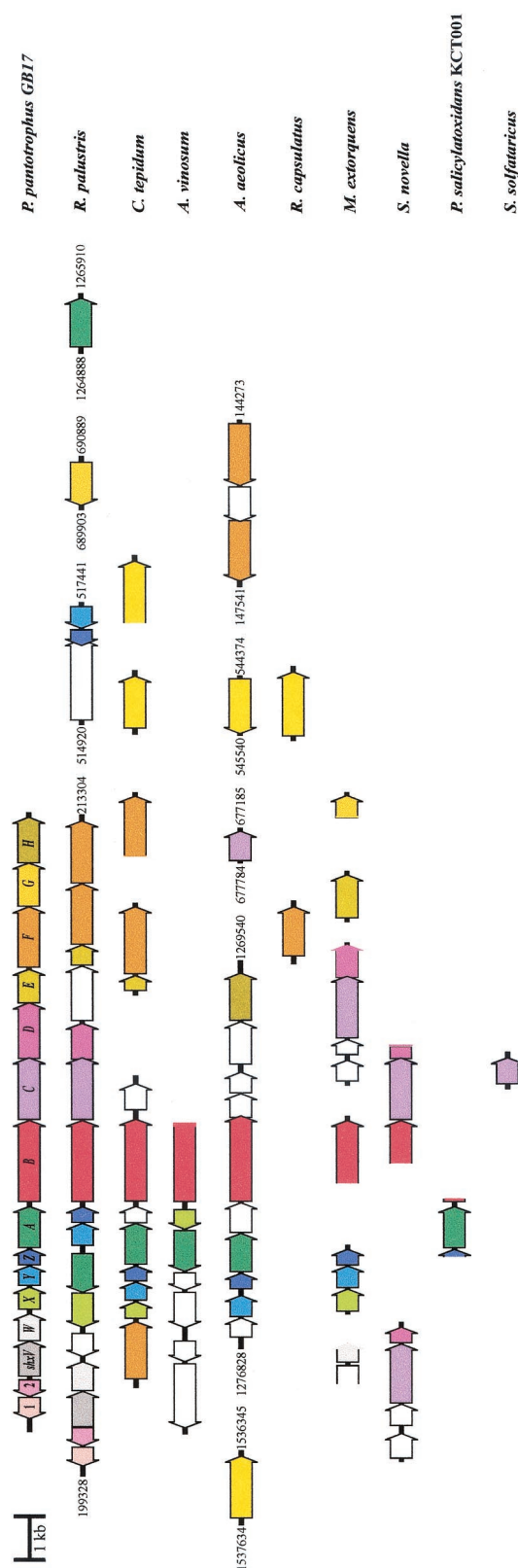


FIG. 2. Schematic map of the *sox* gene cluster of *P. pantotrophus* and identified or putative *sox* genes of other sulfur-oxidizing bacteria. ORFs predicting homologous proteins are indicated by the same color. ORFs that encode proteins not homologous to Sox proteins of *P. pantotrophus* are white. The numbers indicate the positions of putative *sox* genes in the complete genomes. Accession numbers of the ORFs are given in Table 1.

TABLE 1. *sox* genes of *P. pantotrophus* and genes thought to be involved in sulfur oxidation in other thiobacteria

Organism	Gene	No. of amino acids	% Identity to <i>P. pantotrophus</i> ^a	Signal peptide with twin arginine motif	Putative function	Reference(s)	Accession no.
<i>P. pantotrophus</i>	<i>orf1</i>	149		— ^c	Transcriptional regulator	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf685</i>	124	47.1 (104)	— ^c			RRPA00685 ^d
<i>P. pantotrophus</i>	<i>orf2</i>	142		—	Thioredoxin	This study	
<i>R. palustris</i>	<i>orf708</i>	142	41.4 (116)	—			RRPA00708 ^d
<i>P. pantotrophus</i>	<i>shxV</i>	245		—	Reductant transporter	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf618</i>	247	46.4 (237)	—			RRPA00618 ^d
<i>P. pantotrophus</i>	<i>shxW</i>	186		—	Thioredoxin	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf651</i>	197	42.0 (176)	—			RRPA00651 ^d
<i>M. extorquens</i>	<i>orf239^b</i>	119 ^b	47.6 (105)	+ (TRRQFLA)			RMQ00239 ^d
<i>P. pantotrophus</i>	<i>soxX</i>	157		—	Cytochrome <i>c</i>	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf625</i>	247	47.5 (40)	—			RRPA00625 ^d
<i>C. tepidum</i>	<i>orf1709</i>	118	29.7 (37)	—			RCL01709 ^d
<i>A. vinosum</i>	<i>soxX</i>	135	32.5 (80)	—		13	
<i>M. extorquens</i>	<i>orf2551^{b,g}</i>	158 ^{b,g}	47.2 (125)	—			RMQ02551 ^{d,g}
<i>P. pantotrophus</i>	<i>soxY</i>	140		+ (SRREALW)	Sulfur covalently binding protein	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf660</i>	154	44.5 (146)	+ (SRREALA)			RRPA00660 ^d
<i>R. palustris</i>	<i>orf2261</i>	156	34.9 (140)	? (DRRQMLI)			RRPA02261 ^d
<i>C. tepidum</i>	<i>orf1710^s</i>	153 ^s	37.5 (120)	+ (SRRDFCR)			RCL01710 ^{d,g}
<i>A. aeolicus</i>	<i>aq1810</i>	150	30.1 (136)	+ (TRRDFLK)		14	AE000757 ^e
<i>M. extorquens</i>	<i>orf2552</i>	152	56.7 (60)	+ (NRRQALA)			RMQ02552 ^d
<i>P. pantotrophus</i>	<i>soxZ</i>	109		— ^c	Sulfur compound chelating protein	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf674</i>	109	54.1 (109)	— ^c			RRPA00674 ^d
<i>R. palustris</i>	<i>orf2280</i>	111	36.1 (108)	— ^c			RRPA02280 ^d
<i>C. tepidum</i>	<i>orf1711</i>	101	37.0 (81)	— ^c			RCL01711 ^d
<i>A. aeolicus</i>	<i>aq1809</i>	110	36.4 (110)	— ^c		14	AE000757 ^e
<i>M. extorquens</i>	<i>orf2553</i>	109	49.5 (107)	— ^c			RMQ02553 ^d
<i>P. salicylatoxidans</i>	<i>soxZ^b</i>	33 ^b	62.5 (32)	?		43	
<i>P. pantotrophus</i>	<i>soxA</i>	290		—	Cytochrome <i>c</i> (diheme)	20	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf637</i>	273	28.5 (144)	—	Cytochrome <i>c</i> (monoheme)		RRPA00637 ^d
<i>R. palustris</i>	<i>orf2737</i>	341	22.5 (120)	— ^c	Cytochrome <i>c</i> (diheme)		RRPA02737 ^d
<i>C. tepidum</i>	<i>orf1712</i>	286	29.5 (176)	—	Cytochrome <i>c</i> (monoheme)		RCL01712 ^d
<i>A. vinosum</i>	<i>soxA</i>	281	30.0 (207)	—	Cytochrome <i>c</i> (monoheme)	13	
<i>A. aeolicus</i>	<i>aq1807</i>	267	29.2 (178)	—	Cytochrome <i>c</i> (diheme)	14	AE000757 ^e
<i>P. salicylatoxidans</i>	<i>soxA</i>	286	46.0 (276)	—	Cytochrome <i>c</i> (diheme)	43	AJ404005
<i>P. pantotrophus</i>	<i>soxB</i>	564		+ (TRREFIQ)	Sulfate thiol esterase	20, 69	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf525</i>	565	59.8 (565)	+ (SRREFLQ)			RPA00525 ^d
<i>C. tepidum</i>	<i>orf1714^s</i>	584 ^s	47.9 (376)	+ (SRREFLR)			RCL01714 ^{d,g}
<i>A. vinosum</i>	<i>soxB</i>	522 ^b	49.9 (519)	+ (SRREFVR)		13	
<i>A. aeolicus</i>	<i>orf724</i>	592	40.5 (551)	+ (TRRDFFLE)	Quinol-oxidase polypeptide 1	14	AE000757 ^e
<i>M. extorquens</i>	<i>orf2550^b</i>	374 ^b	65.7 (370)	?			RMQ02550 ^d
<i>S. novella</i>	<i>soxB^b</i>	309 ^b	62.3 (308)	?			AF139113 ^e
<i>P. salicylatoxidans</i>	<i>soxB^b</i>	7 ^b	85.7 (7)	+ (SRREF..)		43	
<i>P. pantotrophus</i>	<i>soxC</i>	430		+ (SRR AFLR)	Sulfur dehydrogenase	70	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf545</i>	431	61.3 (408)	+ (NRRRFLG)			RRPA00545 ^d
<i>M. extorquens</i>	<i>orf3760^{b,g}</i>	403 ^{b,g}	45.6 (410)	+ (NRR AFLR)			RMQ03760 ^{d,g}
<i>A. aeolicus</i>	<i>aq979</i>	200	25.2 (119)	— ^c		14	AE000716 ^c
<i>S. novella</i>	<i>soxC</i>	427	61.2 (428)	+ (DRRRFLN)			AF139113 ^e
<i>S. novella</i>	<i>sorA</i>	405	26.5 (385)	+ (NRRQILK)	Sulfite-cytochrome <i>c</i> oxidoreductase subunit A	28	AF154565 ^e
<i>S. solfataricus</i>		209	37.0 (100)	+ (NRRDFLK)		J. van der Oost	
<i>P. pantotrophus</i>	<i>soxD</i>	384		—	Cytochrome <i>c</i>	70	X79242PDSOX ^f
<i>R. palustris</i>	<i>orf644</i>	251	52.7 (201)	—			RRPA00644 ^d
<i>M. extorquens</i>	<i>orf3759^{b,g}</i>	190 ^{b,g}	47.2 (176)	—			RMQ03759 ^{d,g}

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TABLE 1—Continued

Organism	Gene	No. of amino acids	% Identity to <i>P. pantotrophus</i> ^a	Signal peptide with twin arginine motif	Putative function	Reference(s)	Accession no.
<i>S. novella</i>	<i>soxD</i> ^b	84 ^b	55.2 (67)	—			AF139113 ^c
<i>S. novella</i>	<i>sorB</i>	108	60.0 (10)	—	<i>c</i> -type heme subunit of <i>sorA</i>	28	AF154565 ^c
<i>P. pantotrophus</i>	<i>soxE</i>	236		—	Cytochrome <i>c</i>	70	X79242PDSOX/
<i>R. palustris</i>	<i>orf701</i>	139	49.4 (77)	—			RRPA00701 ^d
<i>M. extorquens</i>	<i>orf3227</i>	305	43.0 (107)	—			RMQ03227 ^d
<i>P. pantotrophus</i>	<i>soxF</i>	420		+ (TRRSLIA)	Flavoprotein	51a, 70	X79242PDSOX/
<i>R. palustris</i>	<i>orf554</i>	421	57.1 (422)	+(NRRDVFK)			RRPA00554 ^d
<i>R. palustris</i>	<i>orf566</i>	430	48.1 (420)	+(SRRGMIR)			RPA00566 ^d
<i>C. tepidum</i>	<i>orf1707</i> ^g	430 ^e	40.2 (420)	+(SRRTFNR)			RCL01707 ^{d, g}
<i>C. tepidum</i>	<i>orf258</i>	458	46.3 (382)	+(SRRDFNK)			RCL00258 ^d
<i>A. aeolicus</i>	<i>dhsU</i>	436	30.5 (410)	+(NRRDVFK)	Flavocytochrome <i>c</i> sulfide dehydrogenase	14	AE000679 ^e
<i>A. aeolicus</i>	<i>fccB</i>	417	30.1 (379)	+(DRRNLLL)	Sulfide dehydrogenase, flavoprotein subunit	14	AE000679 ^e
<i>R. capsulatus</i>	<i>orf443</i>	340	26.7 (318)	+(TRRHLAL)			RCL00443 ^d
<i>P. pantotrophus</i>	<i>soxG</i>	303		+(SRRHFLA)	Thiol esterase	51a	X79242PDSOX/
<i>M. extorquens</i>	<i>orf2512</i> ^b	145 ^b	32.7 (113)	?			RMQ02512 ^d
<i>P. pantotrophus</i>	<i>soxH</i>	317		? (MRRLILC)	Thiol esterase	51a	X79242PDSOX/
<i>A. aeolicus</i>	<i>cphA1</i>	326	27.2 (268)	—	beta-Lactamase precursor	14	AE000757 ^e
<i>R. palustris</i>	<i>orf708</i>	142		—	Thiol-disulfide isomerase and thioredoxins		RRPA00708 ^d
<i>A. aeolicus</i>	<i>rhdA2</i>	293		—	Thiosulfate sulfur transferase	14	AE000757 ^e
<i>A. aeolicus</i>	<i>aq1806</i>	206		—		14	AE000757 ^e
<i>A. aeolicus</i>	<i>trxA2</i>	135		—	Thioredoxin	14	AE000757 ^e
<i>C. tepidum</i>	<i>orf717</i> ^b	444 ^b		?	Sulfide-quinone oxidoreductase		RCL00717 ^d
<i>C. tepidum</i>	<i>orf540</i>	390		—	Sulfide-quinone oxidoreductase		RCL00540 ^d
<i>A. aeolicus</i>	<i>sqr</i>	430		—	Sulfide-quinone reductase	14	AE000777 ^e
<i>R. capsulatus</i>	<i>sqr</i>	427		—	Sulfide-quinone reductase	53, 54	X97478 ^f

^a The numbers in parentheses indicate the sizes of the amino acid overlaps.

^b The ORF is incomplete.

^c There is no signal peptide.

^d IGWit database accession number.

^e GenBank database accession number.

^f EMBL data library accession number.

^g Based on our analysis, we concluded that some sequences contain errors which affect the lengths of the ORFs, as follows: in *orf1710* of *C. tepidum* the predicted start is 72 nucleotides upstream of the designated start; in *orf1714* of *C. tepidum* the predicted start is 96 nucleotides downstream of the designated start; in *orf3760* of *M. extorquens* the predicted start is at nucleotide 1838 due to a frameshift around position 1426 upstream of the designated start at nucleotide 1384; in *orf3759* of *M. extorquens* the predicted start is at nucleotide 639 due to a frameshift around position 505 downstream of the designated start at nucleotide 518; and in *orf1707* of *C. tepidum* the predicted start is 90 nucleotides upstream of the designated start.

is increased about 20-fold by 0.3 mM cystine and 2-fold by 0.3 mM cysteine, suggesting that there is a periplasmic oxidation reaction. *shxW* predicts a periplasmic thioredoxin that is unusual with respect to the predicted distribution of β -strands and α -helices, which very likely specify its role either in a redox reaction or for transport of reductant (Bardischewsky and Friedrich, submitted).

The *soxEFGH* genes are located downstream of *soxD* (Fig. 2). These genes are coexpressed with the *sox* structural genes, and thiosulfate-induced formation of SoxFGH has been demonstrated. However, a function could not be determined for these proteins since an in-frame deletion in *soxF* and complementation did not reveal obvious phenotypes of the mutants (51a).

Biochemistry of the Sox system of *P. pantotrophus*. Seven *sox* structural genes code for four proteins. SoxXA, SoxYZ, SoxB, and SoxCD are required for sulfur-dependent cytochrome *c* reduction. SoxXA is a heterodimeric *c*-type cytochrome; SoxX (molecular mass, 14,834 Da) is a monoheme subunit, and SoxA (molecular mass including the heme moieties, 30,452 Da) is a diheme subunit. SoxYZ is composed of SoxY and SoxZ, which have predicted molecular masses of 10,977 and 11,718 Da, respectively. Neither SoxY nor SoxZ contains a cofactor or metal. The molecular mass of SoxY as determined by electrospray mass spectrometry (11,094 Da) differs from the predicted molecular mass of the mature protein by 117 Da (20). Thiosulfate covalently bound to SoxY accounts for 112 Da of the difference, and the higher molecular mass suggests that such an

adduct is formed. In native gradient polyacrylamide gel electrophoresis gels SoxYZ appears as a heterodimer and as a 50-kDa $\alpha_2\beta_2$ heterotetramer, and two forms of the heterodimer that have molecular masses of 29 and 31 kDa are observed (20).

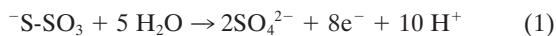
The monomeric SoxB protein contains two manganese atoms (70), and the predicted molecular mass (58,611 Da) is identical to the molecular mass determined by electrospray mass spectrometry. Previous differences between the predicted and determined molecular masses (20) were resolved by analysis of the almost complete primary sequence and the N-terminal 32-pyrroglutamate, which also identified the cleavage site of the signal peptidase (G. Grelle, unpublished data).

SoxCD is an $\alpha_2\beta_2$ heterotetramer (molecular mass, 190,000 Da); SoxC is the molybdenum cofactor-containing subunit (molecular mass, 43,897 Da), and SoxD is the diheme *c*-type cytochrome (molecular mass including the two heme moieties, 38,815 Da) (47).

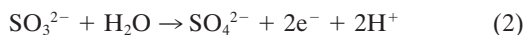
soxE predicts a diheme *c*-type cytochrome (molecular mass of the mature protein, 23,616 Da), and it is thought that SoxE is associated with SoxF (70). *soxF* encodes a 42,832-Da monomeric flavoprotein that includes a flavin moiety, as determined by electrospray mass spectrometry (51a). The primary sequence of SoxF is very similar to the primary sequence of FccB (70), the flavoprotein of flavocytochrome *c*-sulfide dehydrogenase of the phototrophic bacterium *A. vinosum* (71). SoxF does not exhibit sulfide dehydrogenase activity and appears not to be associated with a cytochrome (51a). *soxG* codes for a 29,657-Da polypeptide with two zinc binding motifs. The homodimeric SoxG molecule contains 0.90 atom of zinc per subunit. *soxH* codes for a 32,317-Da protein with a zinc binding motif and another metal binding motif. By using homodimeric SoxH it has been determined that there is 0.29 atom of zinc and 0.20 atom of copper per subunit (51a).

Catalytic properties of the Sox system of *P. pantotrophus*.

The Sox system reconstituted from SoxXA, SoxYZ, SoxB, and SoxCD mediates thiosulfate-, sulfite-, sulfur-, and hydrogen sulfide-dependent cytochrome *c* reduction, and each of the proteins alone is catalytically inactive (20, 51a). Thiosulfate is oxidized by the system according to equation 1, and sulfite is oxidized according to equation 2. Sulfite is also oxidized without SoxCD. This result excludes a function as a sulfite dehydrogenase and suggests a novel function for this molybdoenzyme:



Sulfur-oxidizing enzyme system

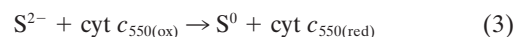


Sox system, sulfite oxidoreductase

Without SoxCD 2 mol of electrons is produced per mol of thiosulfate, and addition of SoxCD increases the yield to eight electrons (20). Since no free intermediate of sulfur oxidation is observed, we suggest that SoxCD acts as a dehydrogenase at a protein-bound sulfur atom, and this protein is designated sulfur dehydrogenase. This suggestion is in accordance with the phenotype of a mutant with an in-frame deletion in *soxC* that is not able to grow lithotrophically with thiosulfate but is still able to oxidize thiosulfate, although at a low rate (51a, 70).

SOX SYSTEMS OF OTHER SULFUR-OXIDIZING BACTERIA

Oxidation of hydrogen sulfide to sulfur. Phototrophic bacteria like *Rhodobacter capsulatus*, *Pelodictyon luteolum*, and some *Chlorobium* species anaerobically oxidize hydrogen sulfide to sulfur in the light. In vitro, hydrogen sulfide is oxidized to sulfur by some phototrophic and chemotrophic bacteria via a flavocytochrome *c*-sulfide dehydrogenase (equation 3) (22, 66) and a sulfide-quinone reductase (equation 4) (53). Flavocytochrome *c* of *A. vinosum* is not required for phototrophic growth with hydrogen sulfide, as shown by disruption of the cytochrome subunit *fccA*, since no obvious phenotype is observed for a *fccA::Ω* mutant (50).



Flavocytochrome *c*-sulfide dehydrogenase



Sulfide-quinone reductase

Therefore, it has been suggested that sulfide-quinone reductase is essential for growth of *A. vinosum* with hydrogen sulfide (50). Sulfide-quinone reductase has been shown to be essential for phototrophic growth of *R. capsulatus* with hydrogen sulfide (53). Hydrogen sulfide is the only sulfur substrate that *R. capsulatus* utilizes for phototrophic growth which is oxidized to sulfur, while *A. vinosum* oxidizes hydrogen sulfide to sulfate (18). Thus, the enzyme systems that oxidize hydrogen sulfide to sulfur and to sulfate are probably different.

SoxF of *P. pantotrophus* is not required for oxidation of reduced inorganic sulfur compounds in vitro (20). Moreover, a mutant with an in-frame deletion in *soxF* grows with thiosulfate, just as the wild-type does (51a). Two flavoprotein homologues have been observed in different genomes of sulfur-oxidizing bacteria (Fig. 2). Since the functions of these homologues are unknown, it is not known if they can complement each other.

Sulfide-quinone reductase has been characterized from *Chlorobium* (55), *R. capsulatus* (54), and the cyanobacterium *Oscillatoria limnetica* (3). Sulfide-quinone reductase activity is also present in *P. pantotrophus* (52), but it does not account for lithotrophic hydrogen sulfide oxidation since an insertional mutant with a mutation in the *soxB* gene is not able to oxidize hydrogen sulfide and thiosulfate (11).

Oxidation of sulfur to sulfate. The first thiosulfate-oxidizing enzyme system analyzed in detail (equation 1) was that of *Paracoccus versutus* (formerly *Thiobacillus versutus* [30]). This system (reviewed in reference 35) is composed of enzyme A (molecular mass, 16,000 Da), enzyme B (60,000 Da), hexameric cytochrome *c*₅₅₁ (260,000 Da) with 43,000-Da subunits, which is intimately associated with sulfite dehydrogenase (44,000 Da), and homodimeric cytochrome *c*_{552.5} (35). An important study has demonstrated that enzyme A binds thiosulfate to sulfite as a competitive inhibitor (40). In spite of differences in the subunit compositions of enzyme A, cytochrome *c*_{552.5}, and cytochrome *c*₅₅₁-sulfite dehydrogenase compared to SoxYZ, SoxXA, and SoxCD, respectively, the similar partial primary sequences of *P. versutus* Sox proteins (20, 69) suggest

that the system is similar to that of *P. pantotrophus* with respect to structure and function (18):



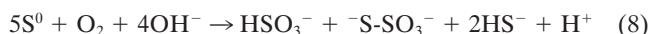
Thiosulfate dehydrogenase



Tetrathionate hydrolase



Trithionate hydrolase



Sulfur oxygenase-reductase

From other sulfur-oxidizing bacteria different enzymes involved in oxidation or hydrolysis of inorganic sulfur compounds have been characterized (equations 5 to 7). The reactions, however, do not involve oxidation of sulfur to sulfate. From *Acidianus brierleyi* a sulfur oxygenase was described (17), and from *Acidianus ambivalens* a sulfur oxygenase-reductase was described (37, 38). The two proteins probably function identically and produce sulfite, hydrogen sulfide, and thiosulfate from sulfur and molecular oxygen (equation 8). No link to energy metabolism has been reported (38), and the significance of a sulfur oxygenase-reductase in energy metabolism is not clear. This enzyme has not been detected in *Bacteria*.

Sulfite oxidoreductase oxidizes sulfite to sulfate (equation 2) and has been characterized from *Starkeya novella* and some members of the *Eukarya* (28, 64). Sulfite oxidoreductase is present in *S. novella* at high specific activity, and its function clearly differs from that of SoxCD. Thiosulfate dehydrogenase oxidizes thiosulfate to tetrathionate (equation 5) and is present in various litho- or phototrophic sulfur-oxidizing bacteria (9, 31). Trithionate hydrolase yields thiosulfate and sulfate. In general, polythionate hydrolysis yields polysulfide sulfate esters $[\text{O}_3\text{S-S-(S-)}_x]^{2-}$, and these esters decompose spontaneously to sulfur and thiosulfate (59).

ANALYSIS OF GENOME SEQUENCES

Proteins homologous to the Sox proteins of *P. pantotrophus* were found in members of the domain *Bacteria* but not in members of the domain *Archaea*. From the complete genome of *Sulfolobus solfataricus* only a periplasmic sulfite dehydrogenase was predicted (J. van der Oost, personal communication). Proteins homologous to the Sox proteins of *P. pantotrophus* were detected in members of the domain *Bacteria*, such as the thermophile *Aquifex aeolicus* (25), the moderately thermophilic green bacterium *Chlorobium tepidum* (67), and the purple bacterium *Rhodopseudomonas palustris* (65). For the facultative methylotroph *Methylobacterium extorquens* (1, 45) essential Sox proteins were deduced from the partial genome sequence (Table 1). Partial *sox* gene clusters with the same order of genes as *P. pantotrophus* were detected in *S. novella* and *Pseudaminobacter salicylatoxidans* KCT001 (Fig. 2), both of which grow with tetrathionate (29, 43). It was predicted that these proteins are located in the periplasm and are transported to the periplasm from the

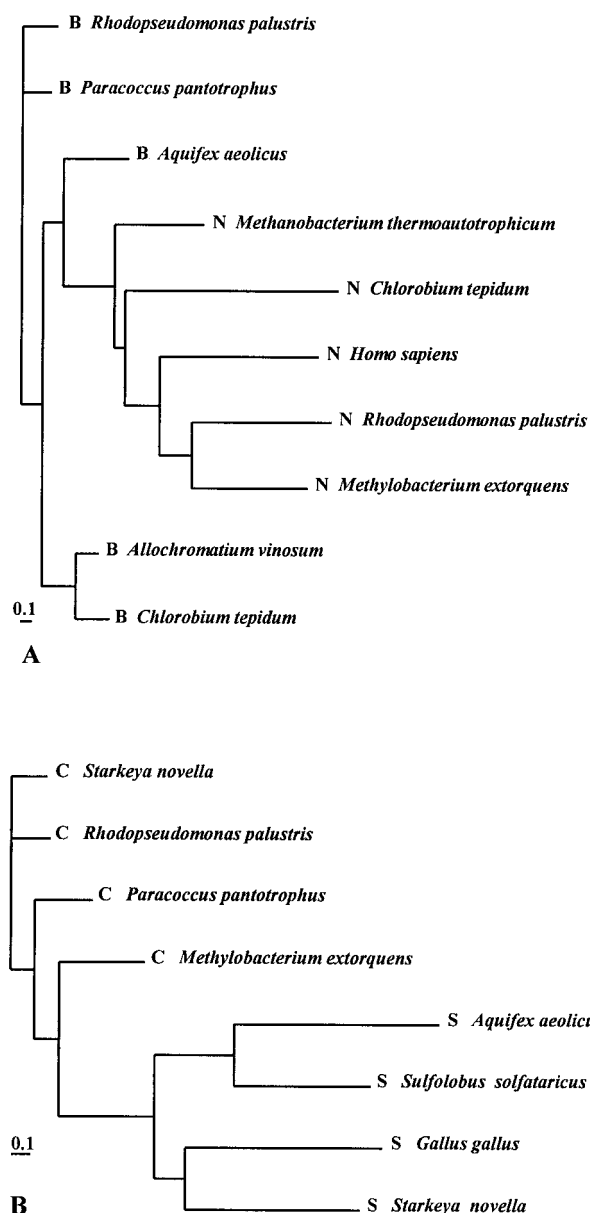


FIG. 3. Phylogenetic relationships of SoxB and SoxC homologous proteins of different bacteria. (A) Proteins that were identified as SoxB homologues (B) and 5'-nucleotidases (N). (B) SoxC (C) and sulfite oxidoreductase (S) homologous proteins. The bar indicates the estimated distance of accepted point mutations per 100 amino acids.

cytoplasm via either the Tat system or the Sec system (6, 18, 68).

Analysis of genome data allows workers to differentiate the function of SoxB and SoxC from the function of their homologues. SoxB proteins exhibit significant identities to 5'-nucleotidases but are distinct from these molecules on a protein phylogenetic tree (Fig. 3A). In view of the differences and since phosphate is not involved in sulfur oxidation but is isosteric to sulfate, we suggest that SoxB proteins function as sulfate thiohydrolases but not as phosphatases.

It has been suggested that SoxC proteins act as dehydrogenases with the protein-bound sulfur atom, while their homo-

logues, sulfite oxidoreductases (like SorA), oxidize free sulfite to sulfate. Both types of enzymes are molybdenum hydroxylases, and the suggested different functions of SorA and SoxC coincide with the separate positions of the molecules on the protein phylogenetic tree (Fig. 3B).

sox genes of phototrophic sulfur-oxidizing bacteria. The *sox* gene cluster of *R. palustris* is similar to that of *P. pantotrophus* and comprises 16 genes equivalent to ORF1, ORF2, *shxVW*, and *soxXYZBCDEF₁F₂*; *soxF₁* and *soxF₂* predict flavoproteins. Two hypothetical genes are in the cluster, while a *soxG* homologue is located at some distance. A second set of *soxA* and *soxYZ* genes and a gene predicting a sulfatase are located outside the *sox* gene cluster (Fig. 2). The *soxA* gene in the *sox* cluster, RRP00637, predicts a monoheme, and the *soxA* gene outside the *sox* gene cluster, RRP02737, predicts a diheme cytochrome (Fig. 2; Table 1).

In a partial genome sequence of *C. tepidum* a *sox* gene cluster comprising eight genes equivalent to *soxF₁XYZAB* followed by a gene predicting a thioredoxin was detected. A hypothetical gene separated *soxB* and *soxA*. Outside the cluster were genes predicting two flavoproteins, one SoxE homologue, and two sulfide-quinone reductases. Genes equivalent to *shxV*, *shxW*, and *soxCD* essential for lithotrophic growth of *P. pantotrophus* with thiosulfate have not been detected in the incomplete genome sequence of *C. tepidum* (Fig. 2).

In *A. vinosum* a partial *sox* gene cluster that included *soxB* and *soxA* was identified by using PCR technology (13). These genes may be essential for sulfur oxidation in this organism since inactivation of *fccA* encoding the cytochrome subunit of flavocytochrome *c*-sulfide dehydrogenase and inactivation of the *aprBA* locus encoding adenosine-5'-phosphosulfate reductase did not affect hydrogen sulfide oxidation (50) or sulfite oxidation (12).

R. capsulatus oxidizes hydrogen sulfide to sulfur, and this activity is linked to sulfide-quinone reductase. Disruption of *sqrA* results in an inability to grow phototrophically with hydrogen sulfide. Thus, this system is clearly different from that described for other sulfur-oxidizing bacteria which oxidize inorganic reduced sulfur compounds to sulfate. From the available genome sequence data for *R. capsulatus* no Sox protein homologue except a flavoprotein has been detected.

sox genes of lithotrophic sulfur-oxidizing bacteria. *A. aeolicus* is an obligately aerobic chemolithotrophic bacterium. This organism requires molecular hydrogen for lithoautotrophic growth and does not grow with thiosulfate alone. However, thiosulfate increases the cellular yield, indicating that it is used for energy conservation (25). The *sox* gene cluster of *A. aeolicus* comprises 10 genes. A gene predicting a thioredoxin is followed by genes equivalent to *soxYZAB* and *soxH*. *soxB* and *soxH* are separated by three ORFs; one of these ORFs predicts a thiosulfate sulfur transferase (rhodanese), while two ORFs have unknown functions. The sulfite dehydrogenase homologue-encoding gene is separated from the *sox* gene cluster of *A. aeolicus*. Also, two genes predicting homologues of the flavoprotein SoxF of *P. pantotrophus* and two putative *sqr* genes are separated from the gene cluster (Fig. 2). The incomplete genomic sequence of *M. extorquens* predicts that there are 10 *sox* genes, 5 of which are incomplete, and these 10 genes are equivalent to *shxV'W'*, *soxXYZ*, *soxB'*, *soxCD'*, *soxE*, and *soxG'*. This finding suggests that *M. extorquens* is a sulfur-

oxidizing bacterium. In fact, this organism utilizes thiosulfate, which increases the cellular yield during mixotrophic growth, and sulfur dehydrogenase (SoxCD) antigens are detected in cells grown with thiosulfate but not in cells grown without thiosulfate (J. Fischer and C. G. Friedrich, unpublished data).

In the tetrathionate-oxidizing organism *S. novella*, *soxC* predicting sulfur dehydrogenase has been identified together with truncated *soxB'* and *soxD'* genes. The gene order *soxB'CD'* is identical to that in *P. pantotrophus* and may indicate that the gene clusters are similar. The *sorAB* genes encode sulfite oxidoreductase and may be separated from *soxB'CD'* (Fig. 2; Table 1). The proposal that there are two thiosulfate-oxidizing systems in *S. novella* was based on the presence of two sulfite dehydrogenase homologues, SorAB and SoxCD (29). The proposed different functions of SoxCD of *S. novella* and SorAB bring into question whether there are two sulfur oxidation pathways and whether SorAB is the only representative of the second pathway. Moreover, induced *S. novella* cells oxidize sulfite at a high rate, which *P. pantotrophus* is unable to do, and the latter organism lacks sulfite oxidoreductase activity (5, 11). In light of the genomic data, a second pathway for thiosulfate oxidation appears to be unlikely, and confirmation of this hypothesis will require inactivation of either SorAB or SoxCD in *S. novella*.

In the tetrathionate-oxidizing organism *P. salicylatoxidans* KCT001 a *soxZ'AB'* gene order is observed (Fig. 2). The predicted diheme *c*-type cytochrome SoxA is highly homologous to SoxA of *P. pantotrophus* and the corresponding cytochromes of other phototrophic or lithotrophic sulfur-oxidizing bacteria (Table 1). A partial *soxZ* gene is located upstream of *soxA*, and downstream a truncated ORF predicts a partial leader peptide of SoxB. The order of these genes is identical to the order in *P. pantotrophus* (Fig. 2). Disruption of *soxA* of *P. salicylatoxidans* KCT001 results in an inability to grow with and to oxidize thiosulfate and tetrathionate (43), demonstrating that SoxA is essential for sulfur oxidation and that polythionates and thiosulfate may be oxidized to sulfate by the same system.

Common Sox proteins of different sulfur-oxidizing bacteria. The different Sox systems are located exclusively in the periplasm. According to the available data, the *sox* genes appear to be clustered, and homologues of SoxA, SoxB, SoxY, and SoxZ are present in phototrophic, lithotrophic, and methylophilic bacteria which oxidize reduced sulfur compounds to sulfate (in *M. extorquens* a SoxA homologue is missing from the incomplete genome sequence). The Sox proteins share the novel motif of SoxY and conserved regions of SoxZ and SoxB. The novel motif (V/I)KV(T/S)(V/I)GGC is located at the C terminus of SoxY (Fig. 4), and the cysteine residue is predicted to bind to the sulfur atom that is oxidized to sulfate. This prediction is based on the difference between the determined molecular mass of SoxY and the molecular mass predicted from the nucleotide sequence and determined from the amino acid sequence (20).

SoxZ proteins contain two signatures, HXM(E/D)(T/S)GXK(D/T) and SX(N/D)PY (Fig. 4). The cysteine residue present in SoxZ of *P. pantotrophus* and *M. extorquens* appears not to be required for linkage of the proteins for cotransport through the cytoplasmic membrane since periplasmic SoxZ proteins from other sources lack this cysteine. Sulfur oxidation in both organisms is strictly aerobic, while sulfur oxidation in

SoxY

ORF	AA
P. p. SoxY	130-VKVTIGGCGG
R. p. 660	144-VKVTIGGCGG
R. p. 2261	146-IKVSVGGCLT
C. t. 1710	142-VKVTIGGCGG
A. a. 729	141-VKVTVGGCG
M. e. 2552	142-VKVTIGGCXR
	:*:~::~*

SoxZ

ORF	AA
P. p. SoxZ	28-HKMEGQQRKD-12-RFTCEL-14-STNPF
R. p. 674	28-HEMESGQQRKD-12-TFTAQF-14-SANPY
R. p. 2280	30-HPMETGLRKT-12-SFIAEA-14-SANPY
C. t. 1711	19-HPMDTGRVKD-12-EVTATI-14-SKDFY
A. a. 728	28-HPMETGLRKT-13-HVDLYF-14-SKNPY
M. e. 2553	28-HTMESGQRKD-12-TFICDL-14-SANPY
	~::~~::~*

FIG. 4. Predicted sulfur binding motifs of SoxY proteins and conserved signatures of SoxZ proteins of different sulfur-oxidizing bacteria. P. p., *P. pantotrophus*; R. p., *R. palustris*; C. t., *C. tepidum*; A. a., *A. aeolicus*; M. e., *M. extorquens*; AA, amino acid sequence.

phototrophic bacteria is anaerobic and sulfur oxidation in *A. aeolicus* is microaerophilic. Some phototrophic bacteria grow lithotrophically in the dark under microaerophilic conditions with reduced sulfur compounds (27, 39). Therefore, the cysteine residue of SoxZ may play a role in coordination of the Sox proteins or in aerobic sulfur oxidation. Some SoxA homologues are either monoheme or diheme cytochromes. The difference may also have significance for aerobic or anaerobic sulfur metabolism in phototrophic sulfur bacteria or for differences in the initial reaction of sulfur oxidation.

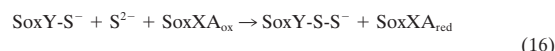
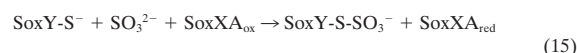
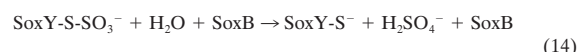
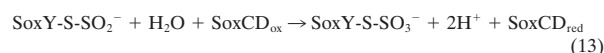
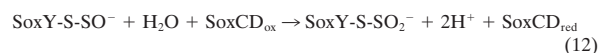
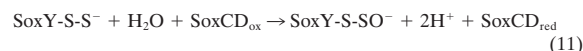
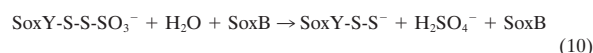
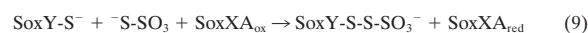
PROPOSAL OF THE MECHANISM OF SULFUR OXIDATION

Functions of the Sox proteins. The reconstituted Sox system of *P. pantotrophus* performs sulfite-, thiosulfate-, sulfur-, and hydrogen sulfide-dependent cytochrome *c* reduction (20, 51a). Oxidation of sulfite by SoxXA, SoxYZ, and SoxB is consistent with the finding that SoxCD does not function as a sulfite dehydrogenase but functions as a sulfur dehydrogenase (20, 51a). The *c*-type cytochrome SoxXA may (i) act as a specific electron mediator, (ii) fuse SoxY with the sulfur substrate in an oxidative reaction (equation 9 [see below]), or (iii) fuse SoxY and SoxZ in aerobic lithotrophs, thus acting as a heme enzyme. The catalytic properties of the Sox proteins of *P. pantotrophus* and the cysteine motif of SoxY suggest a mechanism for sulfur oxidation. The cysteine residue of SoxY is exposed at the C terminus (Fig. 4). Thus, its sulfur atom is located at the tip of the protein and is able to swing to either SoxXA, SoxCD, or SoxB.

Proposed reactions and intermediates. The N-terminal amino acid sequence of SoxZ is highly homologous to that of enzyme A of *P. versutus* (20), suggesting that the function of SoxYZ is identical to the function of enzyme A. Enzyme A of *P. versutus* binds thiosulfate and sulfite. It has been suggested that binding of sulfite occurs at a protein disulfide group (40). By analogy, complexes of the two substrates may be similarly formed by SoxYZ of *P. pantotrophus*. Each subunit contains a

single cysteine whose thiols are able to form a disulfide bond. SoxZ molecules of phototrophic bacteria lack a cysteine residue, and SoxXA may initiate oxidation of thiosulfate to form SoxY-thiocysteine-S-sulfate (equation 9). SoxB would hydrolyze sulfate from the thiocysteine-S-sulfate residue to give S-thiocysteine (equation 10). SoxCD could then oxidize the outer sulfur atom to SoxY-cysteine-S-sulfate (equations 11 to 13). Finally, sulfate may again be hydrolyzed and removed by SoxB to regenerate the cysteine residue of SoxY (equation 14). The sequence of reactions is summarized in Fig. 5.

Sulfite oxidation does not require SoxCD. Sulfite may also be added to SoxY to form SoxY-cysteine-S-sulfate (equation 15), which would be subsequently hydrolyzed by SoxB, yielding sulfate (equation 14). Hydrogen sulfide may be initially oxidized by SoxXA to form an S-thiocysteine residue of SoxY (equation 16) with further oxidation (equations 11 to 13) and hydrolysis (equation 14), and sulfur may be initially oxidized similarly, as proposed for hydrogen sulfide.



Tetrathionate is hydrolyzed by tetrathionate hydrolase to sulfate and thioperoxymonosulfate ($[\text{O}_3\text{S-S-S}]^{2-}$). Its spontaneous decomposition to sulfur and thiosulfate (59) enables the same Sox system to oxidize both products. SoxYZAB proteins

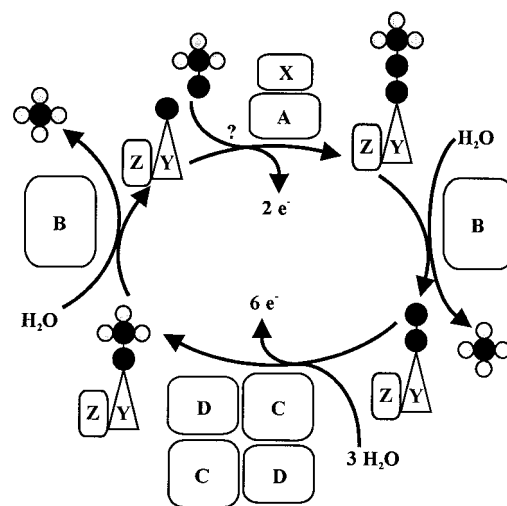


FIG. 5. Model of the sequence of the sulfur oxidation reactions of *P. pantotrophus*.

appear to be crucial for sulfur oxidation and are present in all sulfur-oxidizing bacteria, as deduced from the available data (Fig. 2). In the hyperthermophile *A. aeolicus* a SoxC homologue has not been detected, while SorA appears to be present. Also, ShxVW homologues are missing from the genome of *A. aeolicus*. In this hyperthermophile initiation of sulfur oxidation may occur as it does in other bacteria but may proceed differently, as proposed here.

CONCLUSION

The sulfur-oxidizing enzyme system of *P. pantotrophus* is able to oxidize different reduced inorganic sulfur compounds. It is proposed that the sulfur atom oxidized binds covalently to the cysteine residue of the SoxY protein to form S-thiocysteine. The outer sulfur atom is oxidized by the sulfur dehydrogenase SoxCD, and sulfate is hydrolyzed by the sulfatase SoxB. From available genome sequence data for sulfur-oxidizing bacteria evidence has emerged that similar proteins are present in anaerobic phototrophic and aerobic lithotrophic bacteria but not in the archaeon *S. solfataricus*. Thus, oxidation of sulfur to sulfate may be mediated by very similar systems in bacteria. However, differences may involve the mechanism of linkage of the sulfur atom to be oxidized with SoxY during aerobic and anaerobic sulfur metabolism in lithotrophic and phototrophic bacteria. Also, the systems may differ with respect to the specificities of the actual sulfur substrates.

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We thank Christiane Dahl for making available the nucleotide sequence-predicting *sox* genes of *A. vinosum* prior to publication, John van der Oost for performing the BLAST search of the *S. solfataricus* genome for Sox protein homologues of *P. pantotrophus*, and Erko Stackebrandt for providing the 16S ribosomal DNA sequence of *P. salicylatoxidans* KCT001 prior to publication.

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